STIC-ILL Adonis 25-

From: Schmidt, Mary
Sent: Monday, October 28, 2002 3:51 PM
To: STIC-ILL
Subject: references 09/909,796

Hi, please locate the following references:

Ploszaj et al., Amino acids (Austria), 2000, 19 (2), p483-96.

Stefanelli et al. Biochemical journal (England) May 1, 2000, 347 Pt. 3, p875-80.

Sakagami et al. Anticancer Research (Greece), Jan-Feb. 2000, 20 (1A), p265-70.

Ray et al., American journal of physiology, Cell physiology (US), Mar. 2000, 278 (3), pC480-9.

Bock et al. Radiation research (US), Dec. 1999, 152 (6), p604-10.

Dai et al. Cancer research (US), Oct. 1, 1999, 59 (19), p4944-54.

Bratton et al. Jo. of biological chemistry (US), Oct. 1, 1999, 274 (40), p28113-20.

8 Palyi et al. Anti-cancer drugs (England), Jan 1999, 10 (1), p103-11.

Li et al. Am. journal of physiology , April 1999, 276 (4 Pt. 1), pC946-54.

Ray et al. Am. journal of physiology, Mar. 1999, 276 (3 Pt. 1) pC684-91.

Das et al. Oncology Research (US), 1997, 9 (11-12), p565-72.

Monti et al.. Life Sciences (England), 1998, 62 (9), p799-806.

Lin et al., Experimental cell research, (US), Nov. 25, 1997.

Tome et al. biochemical Journal (England) Dec. 15, 1997, 328 (Pt. 3), p847-54.

Hu et al., Biochemical journal (England), Nov. 15, 1997, 328 (Pt. 1), p307-16.

Tome et al. biological signals (Switzerland), May -Jun 1997, 6 (3), p150-6.

Taguchi et al., Cell biochemistry and function (England), Mar 2001, 19 (1), p19-26.

Camon et al. neurotoxicology (US), Fall 1994, 15 (3), p759-63.

Shinki et al., Gastroenterology (US), Jan 1991, 100 (1), p113-22.

Heston et al. Prostrate (US), 1982, 3 (4), p383-9

Stefanelli et al, biochemical journal (England), Apr. 1, 2001. 355 (pt. 1), p199-206.

Lopez et al., biocell: official journal of the sociedades latinoamericanas de microscopia electronica... et. al. 9Argentina), Dec. 1999, 23 (3), p223-8.

Schipper et al. seminars in cancer biology (US), feb. 2000, 10 (1), p55-68.

Nilsson et al., biochemical journal (England) Mar. 15, 2000, 346 Pt. 3, p699-704.

giuseppina monti m. et al., biochemical and biophysical research commun. (US), Apr. 13, 1999, 257 (2), p460-5.

ratasirayakorn et al, j. of periodontology feb. 1999, 70 (2), p179-84

stabellini et al., Experimental and molecular parhology (US), 1997, 64 (3), p147-55.

Sparapani et al, experimental neurology (US), nov. 1997, 148 (1), p157-66..

Dhalluin et al., carcinogenesis (Eng.), Nov. 1997, 18 (11), p2217-23.

Article title	Effects of methylacetylenic putrescine, an ornithine decarboxylase inhibitor and potential novel anticancer agent, on human and mouse cancer cell lines
Article identifier	0959497399106054
Authors	Pályi_I Kremmer_T Kálnay_A Turi_G Mihalik_R Bencsik_K Boldizsár_M
Journal title	Anti-Cancer Drugs
ISSN	0959-4973
Publisher	Lippincott Williams and Wilkins
Year of publication	1999
Volume	10
Issue	1
Supplement	0
Page range	103-111
Number of pages	9
User name	Adonis
Cost centre	
PCC	$25.00
Date and time	Tuesday, October 29, 2002 10:53:46 PM

Anti-Cancer Drugs 1999, 10, pp. 101–111

Preclinical paper

Effects of methylacetylenic putrescine, an ornithine decarboxylase inhibitor and potential novel anticancer agent, on human and mouse cancer cell lines

István Pályi, Tibor Kremmer, Adrienn Kálnay, Gizella Turi, Rudolf Mihalik,[1] Katalin Bencsik and Mariann Boldizsár

Research Center of Oncology, National Institute of Oncology, 1525 Budapest, Hungary. [1]Institute of Hematology and Blood Transfusion, 1502 Budapest, Hungary.

Sensitivity of several human and mouse cancer cell lines to methylacetylenic putrescine (MAP) was evaluated using clonogenic, sulforhodamine B and cell counting assays. The effects of MAP on cell morphology, cell cycle phase distribution and changes in polyamine metabolism of xenografted MCF-7 and MDA-MB-231 human mammary tumor cells were also investigated. On the basis of IC_{50} values, BHT-101 human thyroid carcinoma cells were the most sensitive (9 μg/ml), followed by P388 mouse lymphoma (32 μg/ml), MCF-7 (48 μg/ml) and MDA-MB-231 (110 μg/ml) human breast carcinoma cell lines. MAP treatment led to accumulation of P388 cells in G_1 phase. At higher doses, the cytoplasm of the cells became vacuolated followed by apoptosis. The foamy cytoplasm may suggest a rare type of cell death (Clarke III type) called non-apoptotic programmed cell death. MAP treatment resulted in a total inhibition of ornithine decarboxylase (ODC) activity with a concomitant decrease of intracellular polyamine (mostly putrescine and spermidine) content in the breast cancer cells, whilst the spermine concentration was shown to increase. MAP proved at least 10 times more potent than the formerly studied DL-α-difluoromethylornithine making it an attractive candidate for clinical testing. [© 1999 Lippincott Williams & Wilkins.]

Key words: Cell cycle, *in vitro* sensitivity, methylacetylenic putrescine, non-apoptotic cell death, ODC activity, polyamine metabolism.

Introduction

Natural poly(oligo)amines are highly basic, with rather simple structures which exhibit definite biological activity. Cumulative data indicate the ubiquitous role of putrescine (Pu), spermidine (Spd), spermine (Spn) and agmatine (Agm) in the regulation and/or modulation of the most basic biosynthetic and reproductive functions (e.g. proliferation, differentiation, etc.) in all living cells under physiological and pathological conditions.[1–5] According to their particular importance in the malignant processes high intracellular Pu and Spd levels have been measured in various experimental and human tumors depending on the rate of cell proliferation.[2,6–13] Due to the high biosynthetic activity of malignant tissues elevated serum concentrations and urinary excretion of polyamines have been reported in cancer patients and regarded as a useful biochemical tumor marker.[7,8,11,14]

Depletion of cellular polyamines using various ornithine and Pu analogs, potential inhibitors of ornithine decarboxylase (ODC, EC 4.1.1.17), the first key enzyme of the polyamine biosynthetic pathway, has opened new perspectives in enzyme-regulated anticancer chemotherapy.[1,9,15–21] Among several active derivatives DL-α-difluoromethylornithine (DFMO, Eflornithine™) has been shown to inhibit cell proliferation and tumor growth *in vitro* and *in vivo*,[13,22–30] and has also been introduced into clinical therapy of human cancers.[17,20,21,31,32] Recently, a promising Pu analog, (2*R*,5*R*)-6-heptyne-2,5-diamine (methylacetylenic putrescine, MAP), has been synthesized and reported to be 10–50 times more active than DFMO.[28,33–38] Both DFMO and MAP altered cell cycle phase distributions by accumulating cells in G_1 phase.[21,26,28] However, both cytostatic and cytotoxic effects were demonstrated depending on the cell line studied.[39] Polyamine analogs were considered to

Correspondence to I Pályi, Research Center of Oncology, National Institute of Oncology, Ráth György str 7–9, 1525 Budapest, Hungary.
Tel: (+36) 1 224-8786; Fax: (+36) 1 224-8786 or (+36) 1 224-8620;
E-mail: palyi@oncol.hu

induce apoptotic cell death, as shown first using a new Spn derivative.[40]

In the present study the effects of MAP on the clonogenicity of P388 mouse lymphoma and of some human cancer cell lines, as well as on the cell cycle distribution and the intracellular polyamine levels of P388 cells were evaluated *in vitro*. Morphological alterations observed in human tumor cell lines, suggesting a special type of programmed cell death, are also described.

Materials and methods

Cell cultures

P388 mouse lymphoma was obtained from I Wodinsky (Arthur D Little, Cambridge, MA) and was established in suspension culture.[41] MCF-7 estrogen receptor (ER)-positive and MDA-MB-231 ER-negative human breast cancer and PC3 prostatic cancer cell lines were obtained from the ATCC (Rockville, MD). BHT-101 human thyroid anaplastic cell line was established in our laboratory.[42]

Drug solutions

MAP was generously supplied by Merrell Research Institute (Strasbourg, France). Fresh MAP solution was made by dissolving the compound in saline or in medium before the experiments.

Clonogenic assay

Cloning efficiency of monolayer cultures was performed as previously described.[42] Briefly, known numbers of exponentially growing cells were plated into triplicate 35 mm Petri dishes (Nunc, Roskilde, Denmark). On the following day the cultures were exposed to different doses of MAP and the MAP was left in contact with the cells throughout the study, and the cultures were incubated in a CO_2 incubator (Heraeus, Hanau, Germany) at 37°C for 10–12 days. The cultures were then rinsed with saline and stained with crystal violet. Colonies containing at least 50 cells were counted. Cloning efficiency of cells growing in suspension was determined as published.[41] The cell suspension, containing the appropriately diluted MAP, was solidified with 0.25% final concentration of agar (Difco, Detroit, MI). Three-dimensional colonies were counted after 10–12 days of incubation under a dissecting microscope.

Absolute cloning efficiency of untreated control cultures was normalized as 100%. Survival of treated cultures was expressed as a fraction of the survival of the control cultures. IC_{50} is the inhibitory drug concentration needed to decrease the survival by 50%.

Antiproliferation studies

The effect of MAP on cell proliferation has been determined by comparing the changes in cell numbers of control and MAP-treated cell populations. MDA-MB-231 or MCF-7 ($2–3 \times 10^5$) cells were seeded into 50 mm Petri dishes and on the following day were exposed to 100–500 μg/ml MAP. The cells were trypsinized on day 2 and the cell numbers were determined using a Neubauer-type hemocytometer.

Flow cytometry

Samples were prepared according to Shapiro.[43] Briefly, 5×10^5 cells were fixed in 70% ethanol and stored at −20°C for a few days. The samples were then centrifuged and washed in phosphate-buffered saline (PBS) solution. After repeated centrifugation, cell pellets were diluted with 1 ml PBS containing 20 μg propidium iodide (PI) (Sigma, St Louis, MO) and 100 μg RNase (Sigma), and were incubated on room temperature for 30 min before measurements. Cell cycle phase distributions were analyzed by measurements of relative DNA content of individual cells using a Cytoron Absolute flow cytometer (Ortho, Raritan, NJ). The quality of the setup was checked by lysed, propidium iodide-stained normal human lymphocytes. The data were analyzed on Cell Cycle software (Ortho).

Cell morphology

MDA-MB-231, BHT-101 and PC3 cells were seeded into Petri dishes, and the morphology of the MAP-treated and control cultures was examined and compared using an Olympus inverted phase contrast microscope. In some cases, fixed and hematoxylin & eosin-stained preparations were also prepared.

Determination of cellular polyamines and ODC activity

Simultaneous determinations of ODC, EC 4.1.1.14 activity and base polyamine levels in cell homogenates were performed by the method of Kvannes and

Flatmark,[44] as modified previously.[45] Polyamines (Pu, Spd and Spn) were extracted from the enzyme reaction and blank (base line level) samples with ice-cold perchloric acid and measured as dansyl derivatives by reversed-phase high-performance liquid chromatography (RP-HPLC). ODC activity was calculated from the amount of putrescine formed in the enzymatic reaction and given in nmol Pu/h/10^6 cells.

Results

Dose survival studies (clonogenic assay)

The effect of MAP on the clonogenicity of P388 mouse lymphoma and BHT-101 human thyroid cancer cells is shown in Figure 1. The first region of the dose-response curves is steep followed by a nearly horizontal 'plateau' portion. Above 75 μg/ml, both curves decline. The IC_{50} values of the P388 and BHT-101 cells were 32 and 9 μg/ml, respectively. The dose-response curves of the two breast cancer lines (Figure 2) are rather different in shape from those of Figure 1. IC_{50} values of MCF-7 and MDA-MB-231 cells were 48 and about 110 μg/ml, respectively. Of the four cell lines studied, the BHT-101 cells were the most sensitive and the MDA-MB-231 cells the least sensitive to MAP. IC_{50} values are summarized on Table 1.

Effect of MAP on cell proliferation

The effect of MAP on the proliferation of ER-positive MCF-7 and ER-negative MDA-MB-231 human breast cancer cell lines was determined by cell counting. No significant retardation of cell proliferation was achieved below 100 μg/ml MAP. The results summarized in Table 2 show a dose-dependent rate of growth retardation, with MCF-7 cells proving more sensitive than MDA-MB-231 cells.

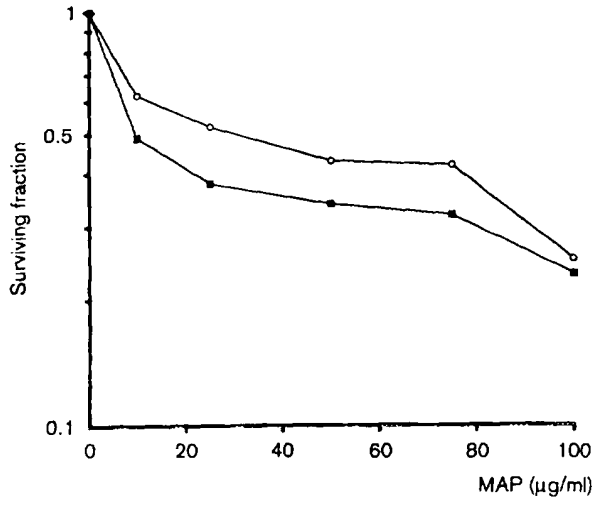

Figure 1. Dose–response curves of P388 mouse lymphoma (○) and BHT-101 human thyroid carcinoma (■) cells treated with MAP continuously.

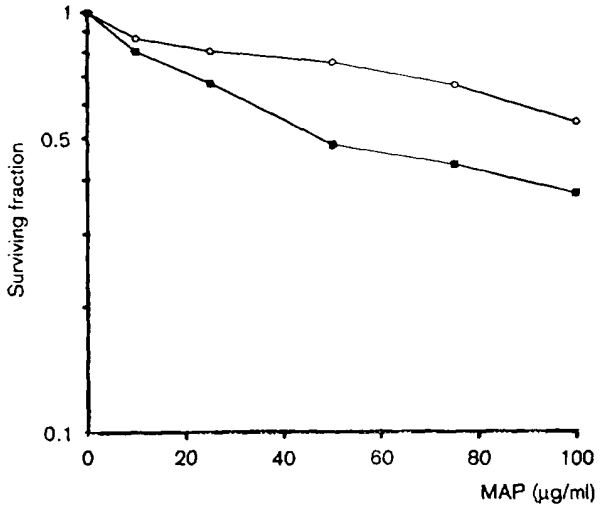

Figure 2. Dose–response curves of MDA-MB-231 (○) and MCF-7 (■) human mammary carcinoma cells treated with MAP continuously.

Table 1. IC_{50} values (mean ± SEM) obtained by treatment with MAP of tumor cell lines in clonogenic assays

Cell line	IC_{50} value (μg/ml)
P388	32 ± 3
BHT-101	9 ± 1
MCF-7	48 ± 4
MDA-MB-231	~110 ± 10

Table 2. Effect of MAP on proliferation of MCF-7 and MDA-MB-231 cells

Doses (μg/ml)	Rate of inhibition (%)	
	MCF-7	MDA-MB-231
100	36 ± 5[a]	21 ± 3
200	42 ± 6	22 ± 3
500	66 ± 8	53 ± 6

[a]Mean values ± SEM at 48 h after treatment.

Effect of MAP on the cell cycle

Concentrations between 10 and 50 μg/ml caused only minimal changes in cell cycle distribution, while those of 75 or 100 μg/ml MAP altered the ratio of cell cycle phases considerably, both at 24 and 48 h following MAP addition (Figure 3 and Table 3). The percent of G_1 phase cells increased from 34 to 47 and 55% at the expense of G_2/M cells. The ratio of S phase cells showed an increase at 24 h and a decrease at 48 h, parallel with the higher concentrations.

Effect of MAP treatment on cell morphology

No characteristic cytomorphological changes were observed below 250 μg/ml MAP. The most typical alteration was vacuolization of the cytoplasm ('foamy cytoplasm') which began to appear in some cells at 250 μg/ml and the vacuoles were small. All cells became heavily vacuolated exposed to 500 μg/ml and the vacuoles were of different sizes. Confluency of the cells ceased and they showed a tendency to

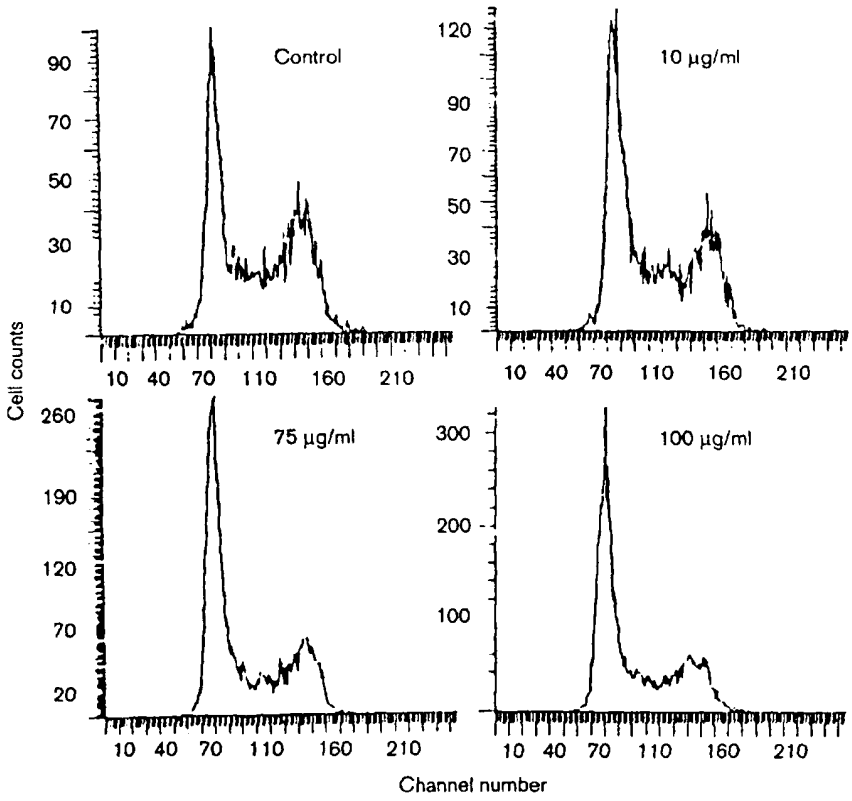

Figure 3. Changes in the cell cycle phase distribution of P388 cells 48 h after exposure to 10, 75 and 100 μg/ml of MAP. The major peak in channel 80 corresponds to G_1 cells.

Table 3. Effect of MAP on the cell cycle phase distribution of P388 mouse lymphoma cells

Doses (μg/ml)	Phase distribution (%)					
	24 h			48 h		
	G_1	S	G_2	G_1	S	G_2
Control	**34**	33	**23**	**34**	38	**28**
10	38	43	19	36	39	25
25	32	45	23	43	34	23
50	34	43	23	42	35	23
75	**47**	36	**17**	**55**	27	**18**
100	41	41	18	55	27	18

round up. A fraction of the cells showed signs of apoptosis, e.g. bleb formation and pyknosis (Figure 4a–c). Long and thin cytoplasmic processes were characteristic by 72 h. At even higher concentrations of MAP (1000 μg/ml), all cells rounded up, began to disintegrate and formed coalesced groups (Figure 4d–f).

Effect of MAP treatment on the ODC activity and polyamine content of MCF-7 and MDA-MB-231 human breast carcinoma cells

Changes in ODC activity and in polyamine content of MCF-7 and MDA-MB-231 human breast cancer cell lines

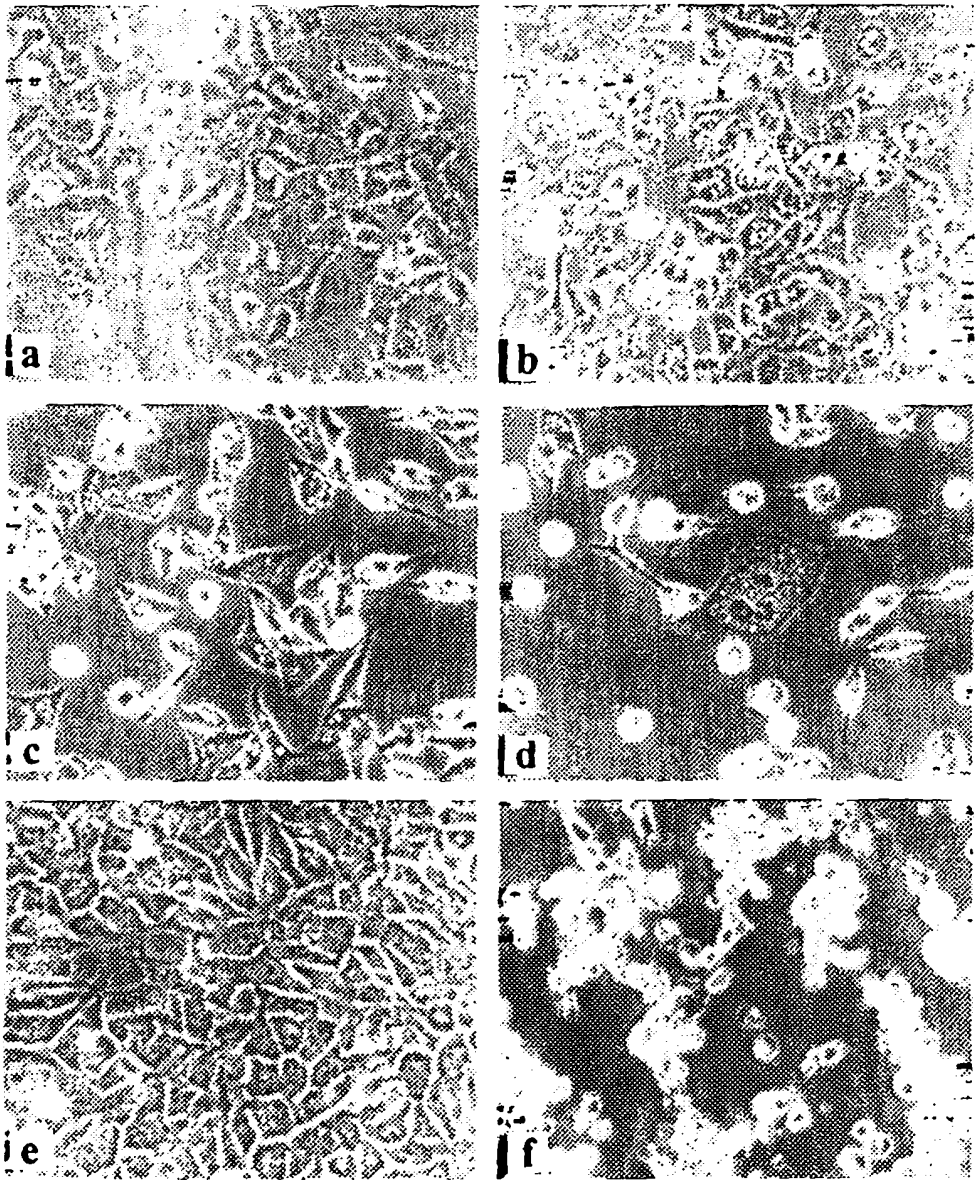

Figure 4. Effect of MAP on cell morphology. (a) Untreated MDA-MB-231 cells with regular morphology and mitotic figures. (b) Cells treated with 500 μg/ml MAP for 24 h. Note the heavily vacuolated cytoplasm and the loose arrangement of the cells. (c) PC3 cells treated with 500 μg/ml MAP for 24 h. The cytoplasm of the cells is filled with vacuoles. Some cells are rounded up, others have long filaments. (d) PC3 cells treated with 1000 μg/ml MAP for 24 h. Most cells rounded up, others have long processes and one giant cell is full of vacuoles. (e) Untreated BHT-101 cells with regular morphology. (f) Cells treated with 1000 μg/ml MAP for 24 h. All cells rounded up producing clusters. Some single cells show apoptosis with bleb formation and freely floating apoptotic bodies are seen. Phase contrast picture of living cells. Magnification: × 160.

induced by a 24 h exposure to 250 μg/ml MAP are summarized in Figure 5. MAP treatment resulted in practically total inhibition of ODC activity in both cell lines. Simultaneously, a decrease in total intracellular polyamine content (mostly in terms of Pu and Spd levels) and a relative increase in Spn concentration was observed. We have found significant differences between the total polyamine base levels of these two cell lines, indicating a higher biosynthetic activity in MDA-MB-231 cells and a higher sensitivity to MAP treatment, first of all, in relation to the changes in Spd content. Retention of Spn induced by MAP in MCF-7 cells also showed a marked contrast to that noted in the MDA-MB-231 line. Similar changes were observed depending on either the MAP concentration (50-500 μg/ml) applied or the time of *in vitro* exposure (24, 48 or 72 h).

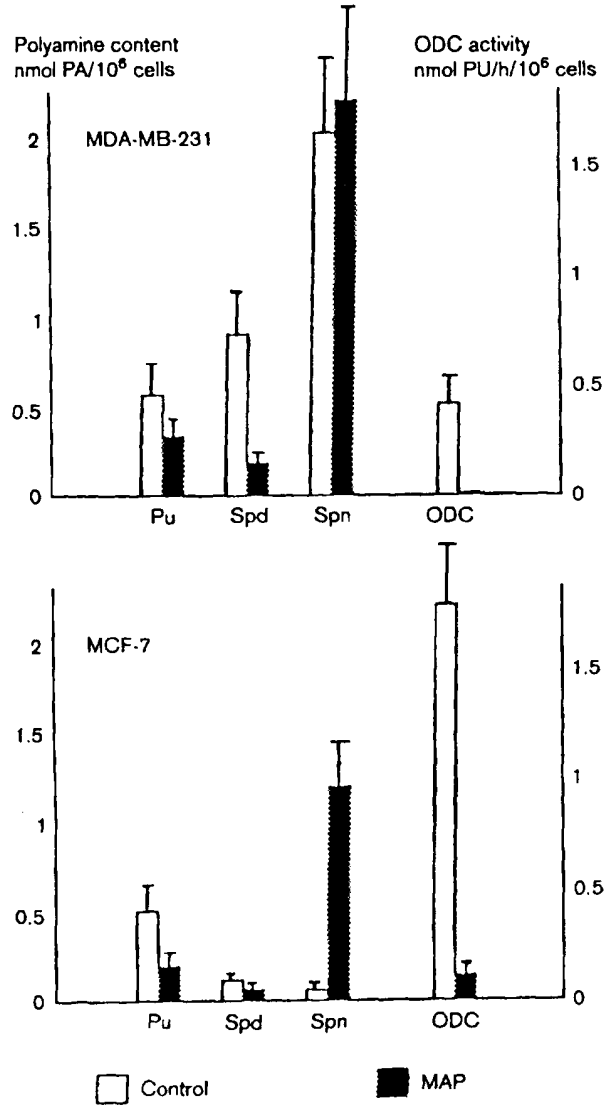

Figure 5. Changes in polyamine content and ODC activity of MCF-7 and MDA-MB-231 breast cancer cells following treatment with 250 μg/ml MAP for 24 h.

Discussion

Our results presented here demonstrate that all cell lines tested were sensitive to MAP. Their sensitivity was, however, dependent on the ER content (MCF-7 versus MDA-MB-231) or on the organ they originated from. The MDA-MB-231 breast cancer cells were the least sensitive, while the BHT-101 thyroid tumor cells were the most sensitive to MAP treatment. This observation supports the significance of the histio-specific toxicity and the disease-oriented anticancer drug discovery screen.[46] In comparison of MAP with the ODC inhibitor DFMO, the former proved to be more potent against malignant human and mouse cell lines. Namely, IC_{50} values of DFMO were 60, 625 and 300 μg/ml for MCF-7, MDA-MB-231 and P388 cells, respectively.[29,30] It means that the difference between DFMO and MAP ranged from slight (20%) to big (10 times). According to other observations, MAP was 20-100,[37] 10-30[33] or 50-300 times[36] more potent than DFMO, depending on the choice of end point, assay and cell type. Bakic *et al.*[28] found only a minimal difference using HL-60 cells and a clonogenic assay. The enhanced growth-inhibitory activity could be due to increased cellular uptake of MAP. The difference in sensitivity to MAP among the various cell lines could also be due to the difference in their doubling times.[36] According to the classic viewpoint, sensitivity is dependent on the rate of proliferation ('proliferation-dependent cytotoxicity'),[47] although we have shown that this view may not be universally valid.[41] Indeed, the slower growing BHT-101 thyroid carcinoma cells were more sensitive to MAP than the fast-growing P388 cells. There was a difference in sensitivity between the two breast cancer cell lines as well, both against DFMO[30] and MAP, with the ER-positive MCF-7 cells being more sensitive. According to Davidson *et al.*[18] there was no clear relationship between either the ER status or the proliferation rate and sensitivity to the spermine analog BESpm. MAP induced an accumulation of cells in G_1 and a decrease of cells in G_2 using P388 mouse lymphoma cells. Similar changes in cells cycle phase distribution following DFMO or MAP treatment were described by several authors.[22,26,37,38,49] This effect can be a consequence of polyamine depletion. The shape of the dose-

response curves, e.g. the plateau portion, also suggested phase-specific effects of the ODC inhibitors.[50] Both compounds exerted cytostatic[39] rather than cytotoxic effects, although the latter were also observed.[24] Whether the effect is cytostatic or cytotoxic is dependent on the cell line employed.[39] In our opinion, it may be concentration dependent as well. At high MAP concentrations, a specific cytomorphological effect appeared in most cell lines, the cytoplasm became heavily vacuolated, 'foamy', followed by cell death. This phenomenon suggests a rare type of programmed cell death as described by Clarke[51] and called non-apoptotic programmed cell death (Clarke III type). Foamy cytoplasm induced by high doses of tyrphostin, a tyrosine kinase inhibitor,[52] has been observed.[53] Programmed cell death induced by a polyamine analog, CPENSpm, was first described by McCloskey *et al.*[40] No other data on programmed cell death elicited by either DFMO or MAP have so far been published. The role of ODC in c-Myc-induced apoptosis has been reviewed by Packham and Cleveland.[54] Polyamines are associated with cell death and the precise regulation of ODC activity in cells has been suggested as preventing polyamine associated cytotoxicity.[55,56] ODC inhibitors are potential anticancer drugs. DFMO has been introduced into clinical trials.[17,20,21] In conclusion, superiority for MAP as a new anticancer drug in comparison to DFMO is expressed in its higher biological activity, its histio-specific selectivity and its induction of programmed cell death.

Acknowledgments

The excellent technical assistance of Vilma Pályi, Judit Szász, Irén Nemes and Edit Szoke is greatly acknowledged.

References

1. Heby O. Role of polyamines in the control of cell proliferation and differentiation. *Differentiation* 1981; **19**: 1–20.
2. Tabor CW, Tabor H. Polyamines. *Annu Rev Biochem* 1984; **53**: 749–90.
3. Seiler N. Polyamines. *J Chromatogr* 1986; **379**: 157–76.
4. Pegg AE. Polyamine metabolism and its importance in neoplastic growth and as a target for chemotherapy. *Cancer Res* 1988; **48**: 759–64.
5. Seiler N, Delcros JG, Moulinoux JP. Polyamine transport in mammalian cells. An update. *Int J Biochem Cell Biol* 1996; **28**: 843–61.
6. Scalabrino G, Ferioli ME. Polyamines in mammalian tumors. *Adv Cancer Res* 1981; **35**: 151–268.
7. Horn Y, Beal SL, Walach N, Lubich WP, Spigel L, Marton LJ. Further evidence for the use of polyamines as biochemical markers for malignant tumors. *Cancer Res* 1982; **42**: 3248–51.
8. Russell DH. Clinical relevance of polyamines. *CRC Crit Rev Clin Lab Sci* 1983; **18**: 261–311.
9. Bondy PK, Canellakis ZN. Polyamines and neoplasia: a review of present knowledge of their function and therapeutic potential. In: Davis W, Maltoni C, Tanneberger ST, eds. *The control of tumour growth and its biological bases*. Berlin: Akademie-Verlag 1983: 258–68.
10. Kremmer T, Selmeci L, Bardócz S, Holczinger L, Bálint S. Polyamine metabolism in P388 leukemia cells and in ascites tumor bearing mice. *Exp Cell Biol* 1984; **52**: 279–85.
11. Horn Y, Beal SL, Walach N, Lubich WP, Spigel L, Marton LJ. Relationship of urinary polyamines to tumor activity and tumor volume in patients. *Cancer Res* 1984; **44**: 4675–8.
12. Seiler N, Knödgen B, Bartholeyns J. Polyamine metabolism and polyamine excretion in normal and tumor bearing rodents. *Anticancer Res* 1985; **5**: 371–8.
13. Seiler N, Sarhan S, Graufferl C, Jones R, Knödgen B, Moulinoux JP. Endogenous and exogenous polyamines in support of tumor growth. *Cancer Res* 1990; **50**: 5077–83.
14. Russell DH, Durie BGM. Polyamines as tumor markers. In: Boelsma E, Rümke Ph, eds. *Tumor markers: impact and prospects, applied methods in oncology 2*. Amsterdam: Elsevier-North Holland 1979: 45–60.
15. Heby O, Oredsson SM, Kanje M. Polyamine biosynthetic enzymes as targets in cancer chemotherapy. *Adv Enz Reg* 1984; **22**: 243–64.
16. McCann PP, Pegg AE, Sjoerdsma A, eds. *Inhibition of polyamine metabolism. Biological significance and basis for new therapies*. New York: Academic Press 1987.
17. Levin VA, Chamberlain MC, Prados MD, *et al.* Phase I–II study of eflornithine and mitoguazone combined in the treatment of recurrent primary brain tumor. *Cancer Treat Rep* 1987; **75**: 459–64.
18. Heston WDW. Prostatic polyamines and polyamine targeting as a new approach to therapy of prostatic cancer. *Cancer Surv* 1991; **11**: 217–38.
19. Quemener V, Blanchard Y, Chamaillard L, Havouis R, Cipolla B, Moulinoux JPh. Polyamine deprivation: a new tool in cancer treatment. *Anticancer Res* 1994; **14**: 443–8.
20. Meyskens FL Jr, Emerson SS, Pelot D, *et al.* Dose-de-escalation chemoprevention trial of α-difluoromethylornithine in patients with colon polyps. *J Natl Cancer Inst* 1994; **86**: 1122–30.
21. Meyskens F, Gerner E, Pelot D, *et al.* A randomized double-blind placebo-controlled phase IIb trial of difluoromethylornithine (DFMO) for colon cancer prevention. *Proc Am Ass Cancer Res* 1997; **38**: 528.
22. Seidenfeld J, Gray JW, Laurence JM. Depletion of 9L rat brain tumor cell polyamine content by treatment with DL-α-difluoromethylornithine inhibits proliferation and the G_1 to S transition. *Exp Cell Res* 1981; **131**: 209–16.

24. Sunkara PS, Chang CC, Prakash NJ, Lichmann PJ. Effect of inhibition of polyamine biosynthesis by DL-α-difluoromethylornithine on the growth and melanogenesis of B16 melanoma *in vitro* and *in vivo*. *Cancer Res* 1985; **45**: 4067–70.
25. Luk GD, Baylin SB. Anchorage dependency effects of difluoromethylornithine cytotoxicity in human lung carcinoma cells. *Cancer Res* 1986; **46**: 1841–8.
26. Luk GD, Abeloff MD, McCann PP, Sjoerdsma A, Baylin SB. Long-term maintenance therapy of established human small cell variant lung carcinoma implants in athymic mice with a cyclic regimen of difluoromethylornithine. *Cancer Res* 1986; **46**: 1849–53.
27. Seidenfeld J, Block AL, Komar KA, Naujokas MF. Altered cell cycle phase distributions in cultured human carcinoma cells partially depleted polyamines by treatment with difluoromethylornithine. *Cancer Res* 1986; **46**: 47–53.
28. Kremmer T, Boldizsár M, Holczinger L. *In vitro* effect of DL-α-difluoromethylornithine (DFMO) on the polyamine and nucleotide phosphate metabolism in P388/S leukemia cells. *Exp Cell Biol* 1986; **54**: 8–15.
29. Bakic M, Chan D, Freireich EJ, Marton LJ, Zwelling LA. Effect of polyamine depletion by α-difluoromethylornithine or (2*R*,5*R*)-6-heptyne-2,5-diamine on drug-induced topoisomerase II-mediated DNA cleavage and cytotoxicity in human and murine leukemia cells. *Cancer Res* 1987; **47**: 6437–43.
30. Kremmer T, Pályi I, Holczinger L, *et al*. Changes in the polyamine and nucleotide metabolism of P388 leukemia cells treated with DL-α-difluoromethylornithine in culture. *Exp Cell Biol* 1988; **56**: 131–7.
31. Kremmer T, Pályi I, Daubner D, *et al*. Comparative studies on the polyamine metabolism and DFMO treatment of MCF-7 and MDA-MB-231 breast cancer cell lines and xenografts. *Anticancer Res* 1991; **11**: 1807–14.
32. Lipton A, Harvey HA, Glenn J, *et al*. A phase I study of hepatic arterial infusion using difluoromethylornithine. *Cancer* 1989; **63**: 433–7.
33. Boiko I, Mitchell MF, Hu W, Malpica A, Pandey D, Hittelman WN. High mean DNA content and EGFR expression predict poor response of CIN 3 lesions to chemoprevention by difluoromethylornithine (DFMO). *Proc Am Ass Cancer Res* 1997; **38**: 262.
34. Danzin G, Casara P, Claverie N, Metcalf BW, Jung MJ. (2*R*,5*R*)-6-heptyne-2,5-diamine, an extremely potent inhibitor of mammalian ornithine decarboxylase. *Biochem Biophys Res Commun* 1983; **116**: 237–43.
35. Bartholeyns J, Mamont P, Casara P. Antitumor properties of (2*R*,5*R*)-6-heptyne-2,5-diamine, a new potent enzyme-activated irreversible inhibitor of ornithine decarboxylase in rodents. *Cancer Res* 1984; **44**: 1972–7.
36. Mamont PS, Siat M, Joder-Ohlenbusch AM, Bernhardt A, Casara P. Effects of (2*R*,5*R*)-6-heptyne-2,5-diamine, a potent inhibitor of L-ornithine decarboxylase, on rat hepatoma cells cultured *in vitro*. *Eur J Biochem* 1984; **142**: 457–63.
37. Pera PJ, Kramer DL, Sufrin JR, Porter CW. Comparison of the biological effect of four irreversible inhibitors of ornithine decarboxylase in two murine lymphocytic leukemia cell lines. *Cancer Res* 1986; **46**: 1148–54.
38. Milam KM, Deen DF, Marton LJ. Cell proliferation and polyamine metabolism in 9L cells treated with (2*R*,5*R*)-6-heptyne-2,5-diamine or α-difluoromethylornithine. *Cell Tissue Kinet* 1989; **22**: 269–77.
39. Adlakha RC, Ashorn C, Wagle J, Nishioka K, Freireich EJ. Differential effects of (2*R*,5*R*)-6-heptyne-2,5 diamine, a potent inhibitor of polyamines on the cell cycle traverse of normal and transformed cells. *Proc Am Ass Cancer Res* 1989; **30**: 586.
40. Sunkara PS, Baylin SB, Luk GD. Inhibitors of polyamine biosynthesis, cellular and *in vitro* effects of tumor proliferation. In: McCann PP, Pegg AE, Sjoerdsma A, eds. *Inhibition of polyamine metabolism*. Orlando: Academic Press 1987: 121–140.
41. McCloskey DE, Casero RA Jr, Woster PM, Davidson NE. Induction of programmed cell death in human breast cancer cells by an unsymmetrically alkylated polyamine analogue. *Cancer Res* 1995; **55**: 3233–6.
42. Pályi I. Survival responses to new cytostatic hexitols of P388 mouse and K562 human leukemia cells *in vitro*. *Cancer Treat Rep* 1986; **70**: 279–84.
43. Pályi I, Péter I, Daubner D, Vincze B, Lorincz I. Establishment, characterization and drug sensitivity of a new anaplastic thyroid carcinoma cell line (BHT-101). *Virchows Archiv B Cell Pathol Mol Pathol* 1993; **63**: 263–9.
44. Shapiro HM. *Practical flow cytometry*. New York: Alan R Liss 1988.
45. Kvannes J, Flatmark T. Rapid and sensitive assay of ornithine decarboxylase activity by high-performance liquid chromatography of the *o*-phthalaldehyde derivative of putrescine. *J Chromatogr* 1987; **419**: 291–5.
46. Kremmer T, Boldizsár M, Paulik E. Application of high-performance liquid chromatographic methods in the monitoring of enzyme-targeted chemotherapy. *Chromatographia* 1988; **26**: 423–8.
47. Shoemaker RH, Monks A, Alley MC, *et al*. Development of human tumor cell line panels for use in disease-oriented drug screening. In: Hall T, ed. *Prediction of response to cancer chemotherapy*. New York: Alan R Liss 1988: 265–85.
48. Van Putten LM. Are cell kinetic data relevant for the design of tumour chemotherapy schedules? *Cell Tissue Kinet* 1974; **7**: 493–504.
49. Davidson NE, Mank AR, Prestigiacomo LJ, Bergeron RJ, Casero RA Jr. Growth inhibition of hormone-responsive and -resistant human breast cancer cell in culture by N^1, N^{12}-Bis(ethyl)spermine. *Cancer Res* 1993; **53**: 2071–5.
50. Ask A, Persson L, Oredsson SM, Heby O. Synergistic antileukemic effect of two polyamine synthesis inhibitors. Host survival and cell-cycle kinetic analysis. *Int J Cancer* 1986; **37**: 465–70.
51. Bruce WR, Meeker BE, Valeriote FA. Comparison of the sensitivity of normal hematopoietic and transplanted lymphoma colony-forming cells to chemotherapeutic agents administered *in vivo*. *J Natl Cancer Inst* 1966; **37**: 233–45.
52. Clarke PGH. Developmental cell death: morphological diversity and multiple mechanisms. *Anat Embryol* 1990; **181**: 195–213.
53. Levitzki G, Gazit A. Tyrosine kinase inhibition: an approach to drug development. *Science* 1995; **267**: 1782–8.

53. Szende B, Kéri Gy, Szegedi Zs, *et al.* Tyrphostin induces non-apoptotic programmed cell death in colon tumor cell. *Cell Biol Int* 1995; **19**: 903–11.
54. Packham G, Cleveland JL. C-Myc and apoptosis. *Biochim Biophys Acta* 1995; **1242**: 11–28.
55. Coffino P, Poznanski A. Killer polyaminase? *J Cell Biochem* 1991; **45**: 54–8.
56. Morris DR. New perspectives on ornithine decarboxylase regulation. Prevention of polyamine toxicity is the overriding theme. *J Cell Biochem* 1991; **46**: 102–5.

(Received 23 July 1998; revised form accepted 18 August 1998)